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Neoichnological study of two species of burrowing darkling beetles (Coleoptera: Tenebrionidae) from larval to adult stages

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ABSTRACT

Beetles (Coleoptera) are a critical component of terrestrial ecosystems filling numerous ecological roles. Soil-dwelling beetles play an important role in pedogenesis through the production of biogenic structures throughout their life cycle. Given their life habits, beetle trace fossils should be abundant in the fossil record and a number of ichnotaxa have been attributed to beetles, yet there is still much to learn about their trace-making techniques and the variety of morphologies of traces they can produce throughout their life cycle. The Tenebrionidae (darkling beetles) is a common, globally distributed group that includes many soil-dwelling species from a variety of environments. The purpose of this project was to gain a better understanding of biogenic structures produced during each life stage of two species of burrowing darkling beetles, *Zophobas morio* F. and *Tenebrio molitor* L. Single to multiple specimens of each species were placed in variably sized enclosures filled with massive and layered sediment with and without layers of food. Biogenic structures produced by the two species were similar, but distinct between each life stage and associated with different behaviors. Observed biogenic structures are comparable to *Palaeophycus, Planolites, Fictovichnus, Rebuffoichnus, Pallichnus, Taenidium, Skolithos,* and *Conichnus* depending on the life stage. Neoichnological studies such as this can aid in the understanding of biogenic structures in the fossil record. Being able to effectively link continental traces to trace makers will ultimately improve our understanding of the composition of ancient terrestrial ecosystems.

KEYWORDS

Bioturbation; continental; darkling beetle; insect; neoichnology; paleoecology; paleoenvironment; soil

Introduction

Beetles (Coleoptera) are a diverse and numerous group of insects that are a critical component of terrestrial ecosystems filling numerous ecological roles in a variety of subenvironments across the majority of continental environments (Marshall, 2018; Zhang, 2011). In modern ecosystems, beetles range from being scavengers, saprophytic, carnivorous, herbivorous, and frugivorous (Coleman et al., 2004). This diversity and abundance makes beetles a good candidate for understanding ecosystem dynamics in both the modern and the ancient. Soil-dwelling beetles play an important role in pedogenesis through the production of biogenic structures during their larval, pupal, and adult life stages (Lavelle & Spain, 2001). While the abundance of soil-dwelling beetles is high in modern ecosystems, their body fossils are relatively rare (Smith & Marcot, 2015). Given their semi-fossorial to fossorial life habits, beetle trace fossils should be abundant in the fossil record and useful proxies for their presence in paleoecosystems. However, despite significant research on beetle traces, especially those of dung beetles (Genise, 2017), there is still much to learn about the trace-making techniques and the variety of morphologies of biogenic structures that can be produced by beetles. Of great importance is the direct observation of the burrowing techniques and behaviors that result in these biogenic structures as well as a recognition of the pattern of changing trace production through their entire life cycle. The possibility to directly observe and identify these patterns are the major values of laboratory-based neoichnology.

The Tenebrionidea (darkling beetles) are a common, diverse, and globally distributed group of extant beetles that includes soil-dwelling species present in many different habitats, but are most closely associated with dry environments such as steppes and deserts (Marshall, 2018; Nabozhenko, 2019). In North America, darkling beetles are one of the most

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common macro-invertebrates in semiarid to arid ecosystems (Crawford, 1981). Among them the Subfamily Tenebrioninae includes beetles that typically live within rotting wood, fungi, and soils of forests, woodlands, grasslands, steppes, and deserts (Marshall, 2018). The Tenebrioninae make up 28 of the 122 described fossil darkling beetle species, occurring in Europe, North America, and South America, making them one of the most diverse sub-families of darkling beetles in the fossil record (Nabozhenko, 2019). In addition to the prevalence of this subfamily in the fossil record, *Zophobas morio* F. and *Tenebrio molitor* L. were selected to be studied because they are easy to acquire and care for in laboratory conditions. They also have relatively rapid life cycles making them ideal specimens for the study of their traces from their larval to adult stages. *Zophobas morio* and *T. molitor* have a nearly global distribution due to the use of their larvae as food sources, but were originally present in Eurasia (*T. molitor*) and South America (*Z. morio*) (Marshall, 2018). In their natural settings, larvae of both species burrow within the substrate in woodlands and then, after pupation, adults live either on the surface or underneath logs or rocks during their adult stage (Cotton, 1927; Smith et al., 2015). The larvae of some species of Tenebrioninae occur within three major soil textural classes: clay, loam, and sand (Calkins & Kirk, 1975). *Zophobas morio* and *T. molitor* are generalist omnivores that feed on decaying plant material, fresh plant material, decaying insects, and fungi in their natural settings (Cotton, 1927; Marshall, 2018). Due to anthropogenic influences, today these species are commonly found in association with stored grain as pests.

Darkling beetles have a complete life cycle consisting of four life stages (egg, larva, pupa, adult) each with distinct morphologies and behaviors (Cotton, 1927; Marshall, 2018) (Figure 1). During their larval stages, darkling beetles appear 'worm like', having radial segments connected across the thorax and the abdomen and six legs attached to the thorax (Marshall, 2018). Towards the end of the larval stage, the larvae construct a chamber for pupation. The exoskeleton changes significantly during pupation and pupae are mostly immobile unless disturbed. Metamorphosis into the adult stage typically occurs after 10–20 days in these species.

In general, insects have a low preservation potential as body fossils due to a lack of biomineralized hard parts. Coleopterans are not an exception, but they do have a higher preservation potential compared to other orders of insects due to their strong exoskeleton (Smith & Marcot, 2015). Fossil specimens of beetles primarily come from amber and lacustrine shale (Kirejtshuk, 2020). Occurrence data (Smith & Marcot, 2015) and molecular age dating (Hunt et al., 2007; McKenna et al., 2019) suggest that beetles first appeared in the Carboniferous or Permian, with diversity spikes in the Early Jurassic and Early Cretaceous. Occurrence data of body fossils have been the main source of data for clarifying beetle ranges and the timing of radiations. While their fossil record appears adequate enough to make many assumptions about the life history of beetles, additional data from the trace fossil record can potentially aid in further clarifying ranges and radiations as well as the ecological roles of beetles through time.

Beetle traces are abundant in both modern and ancient ecosystems (Genise, 2017; Genise et al., 2000), thus, making beetles a good candidate for ichnological studies. The diversity of beetles and the environments they inhabit suggests a significant amount of variability in the types of traces that they could produce. The four-stage life cycle of beetles results in significant alterations in their body form and behaviors, thus, changing the morphology of beetle traces throughout their life. In addition, environmental factors can change burrow morphology due to variations in trace maker behavior and substrate type (Buatois & Mángano, 2011). All of these factors combined (varying substrates, changing body forms, changing behaviors) can lead to a diversity of traces produced by the same organism over its lifespan.

Neoichnological studies provide context for the morphology of trace fossils (Bromley, 1996). Comparing traces from neoichnological studies to trace fossils helps in understanding both the behavior and organism responsible for the traces as well as how the environment around the trace maker influences the resulting trace morphology (Bromley, 1996). Previous neoichnological studies focused on the morphology of the biogenic structures produced by soil-dwelling beetles have provided context on some of the types of traces beetles may produce in terrestrial environments, but many typically only within a single life stage (e.g., Brussaard, 1985; Brussaard & Runia, 1984; Clark & Ratcliffe, 1989; Counts & Hasiotis, 2009; Genise, 2017; Mikus & Uchman, 2013; Ratcliffe & Fagerstrom, 1980; Smith & Hein, 1971). Considering ichnofossils produced by beetles when establishing major events in their life history would help clarify some of the uncertainties that arise when using the body fossil record or low resolution molecular age dating estimates.

The goal of this study was to describe the biogenic structures produced during each life stage of two

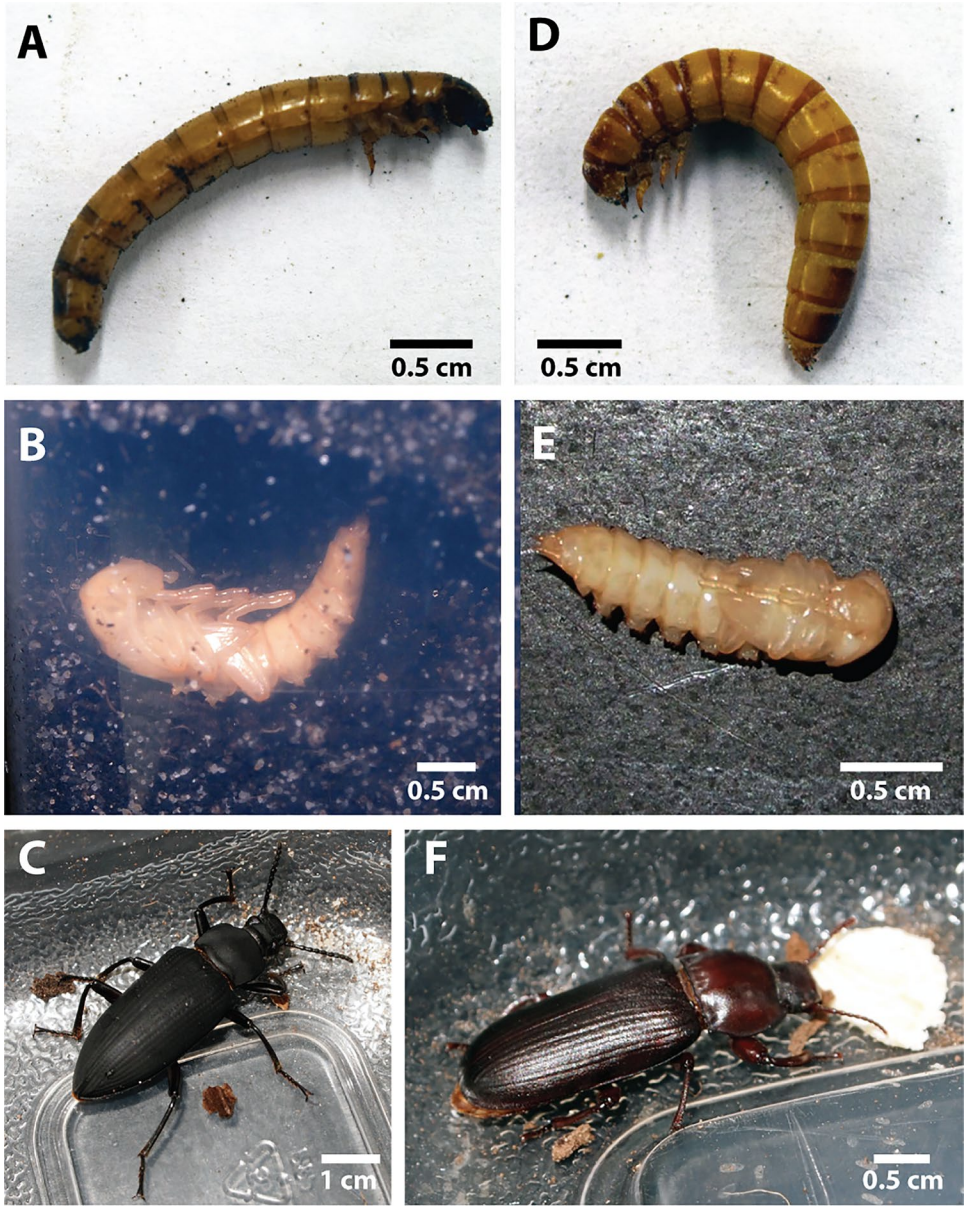

Figure 1. Developmental stages of the studied darkling beetle species. In the left column, *Zophobas morio* stages: (A) larva, (B) pupa, and (C) adult; in the right column, *Tenebrio molitor* stages: (D) larva, (E) pupa, and (F) adult.

species of darkling beetles, *Tenebrio molitor* L. and *Zophobas morio* F. Multiple experimental trials under laboratory conditions were conducted, each with different environmental parameters related to sediment composition, presence of food, and amount of available space.

Materials and methods

Four experiments (Experiments 1–4) were conducted using a total of 48 larvae of *Tenebrio molitor* and 84 larvae of *Zophobas morio*, each placed on the surface of a sediment-filled enclosure and removed once they emerged at the surface in the adult stage (Table 1). All larvae were acquired from a pet supply company. Precise instar stage was not known, but body lengths corresponded to the 15th–20th instars (Park et al., 2014). The *T. molitor* larvae used at the start of the experiments had a mean length of 3 cm and mean width of 0.3 cm. Adult *T. molitor* obtained at the end of the experiments had a mean length of 1 cm and a mean width of 0.5 cm. The *Z. morio* larvae used at the start of the experiments had a mean length of 4 cm and mean width of 0.5 cm. Adult *Z. morio* obtained at the end of the experiments had a mean length of 2.5 cm and a mean width of 0.7 cm.

In all four experiments, the experimental enclosures kept in a darkened laboratory where the air

Table 1. Experiment design for Experiments 1–4.

Experiment 1						
Species	Number of specimens	Sediment composition	Tank size (L)	Temperature range (°C)	Sediment depth (cm)	Food availability
T. molitor	8	15/50/35 (Sa/C/Or)	6	21–24	20	No food
T. molitor	8	15/50/35 (Sa/C/Or)	6	21–24	20	Food
Z. morio	8	15/50/35 (Sa/C/Or)	6	21–24	20	No food
Z. morio	8	15/50/35 (Sa/C/Or)	6	21–24	20	Food
Experiment 2						
Species	Number of specimens	Sediment composition	Tank size (L)	Temperature range (°C)	Sediment depth (cm)	Food availability
T. molitor	8	15/50/35 (Sa/C/Or)	14	21–24	20	No food
T. molitor	8	15/50/35 (Sa/C/Or)	14	21–24	20	Food
Z. morio	8	15/50/35 (Sa/C/Or)	14	21–24	20	No food
Z. morio	8	15/50/35 (Sa/C/Or)	14	21–24	20	Food
Experiment 3						
Species	Number of specimens	Sediment composition	Tank size (L)	Temperature range (°C)	Sediment depth (cm)	Food availability
T. molitor	8	15/50/35 (Sa/C/Or) and 60/25/15 (Sa/C/Or)	6	21–24	20	No food
T. molitor	8	15/50/35 (Sa/C/Or) and 60/25/15 (Sa/C/Or)	6	21–24	20	Food
Z. morio	8	15/50/35 (Sa/C/Or) and 60/25/15 (Sa/C/Or)	6	21–24	20	No food
Z. morio	8	15/50/35 (Sa/C/Or) and 60/25/15 (Sa/C/Or)	6	21–24	20	Food
Experiment 4						
Species	Number of specimens	Sediment composition	Tank size (L)	Temperature range (°C)	Sediment depth (cm)	Food availability
Z. morio	36	15/50/35 (Sa/C/Or) and 60/25/15 (Sa/C/Or)	38	21–24	20	No food

Sa: sand; C: clay; Or: organic matter.

temperature was maintained at 21–24 °C, monitored with digital thermometers. The enclosures were sprayed with water three times per week to maintain a sediment moisture of 25–35% which was monitored with an Aquaterr EC-300 multimeter.

In Experiment 1, each larva of *Z. morio* and *T. molitor* ($n = 16$ per species) was placed on the sediment surface in a single 6 L (17 cm × 27.5 cm × 7.5 cm) plastic enclosure filled with a 20 cm deep, massive, slightly compacted sediment composed of 15% sand, 50% clay, and 35% organic matter (Sa/C/Or; Figure 2A, Table 1). The sediment composition produced an organic-rich loam that was similar to the soil composition found in environments occupied by most Tenebrioninae in natural conditions (Cotton, 1927; Smith et al., 2015). Two sets of enclosures were prepared for each species, one with and one without included layers of food (Table 1). The food layers consisted of three layers of thinly sliced apples placed at 5, 10, and 15 cm above the base of the enclosure to determine the effect of abundant, concentrated food (i.e., decaying organic matter) on burrowing behavior and trace morphology. The objective of Experiment 1 was the observation of the burrowing techniques, morphology of biogenic structures produced, general behaviors associated with burrows, and the potential preservation of traces made during the larval, pupal, and/or adult stages.

Experiment 2 was conducted in the same manner as Experiment 1, but using 14 L (36 cm × 21 cm × 21 cm) enclosures to observe if the behaviors and trace morphologies differed with more available space (Figure 2B, Table 1). The same sediment composition and depth was used as in Experiment 1, as well as the preparation of two sets of eight enclosures for each species, one with and one without food layers.

In Experiment 3, each larva of *Z. morio* and *T. molitor* ($n = 16$ per species) was placed on the sediment surface in a 6 L (17 cm × 27.5 cm × 7.5 cm) plastic enclosure filled with 20 cm of sediment consisting of 1 cm layers of a sediment composed of 15% sand, 50% clay, and 35% organic matter alternating

Table 2. Ranges and means of the dimensions of burrows and chambers produced by *T. molitor* and *Z. morio* in each life stage and the time spent in each life stage.

	Width (mm)	Mean width (mm)	Height (mm)	Mean height (mm)	W/H ratio	Depth (mm)	Mean depth (mm)	Duration (days)
Larva burrows								
T. molitor	1–4	3				1–120	30	12–25
Z. morio	2–8	5				1–200	70	12–25
Pupation chambers								
T. molitor	15–30	22	7–25	15	1.83	10–100	30	13–21
Z. morio	10–50	33	10–30	18	1.93	40–200	120	13–21
Adult exit burrows								
T. molitor	6–12	10						2–5
Z. morio	13–24	15						2–5

Columns without values indicate the measurement is not applicable.

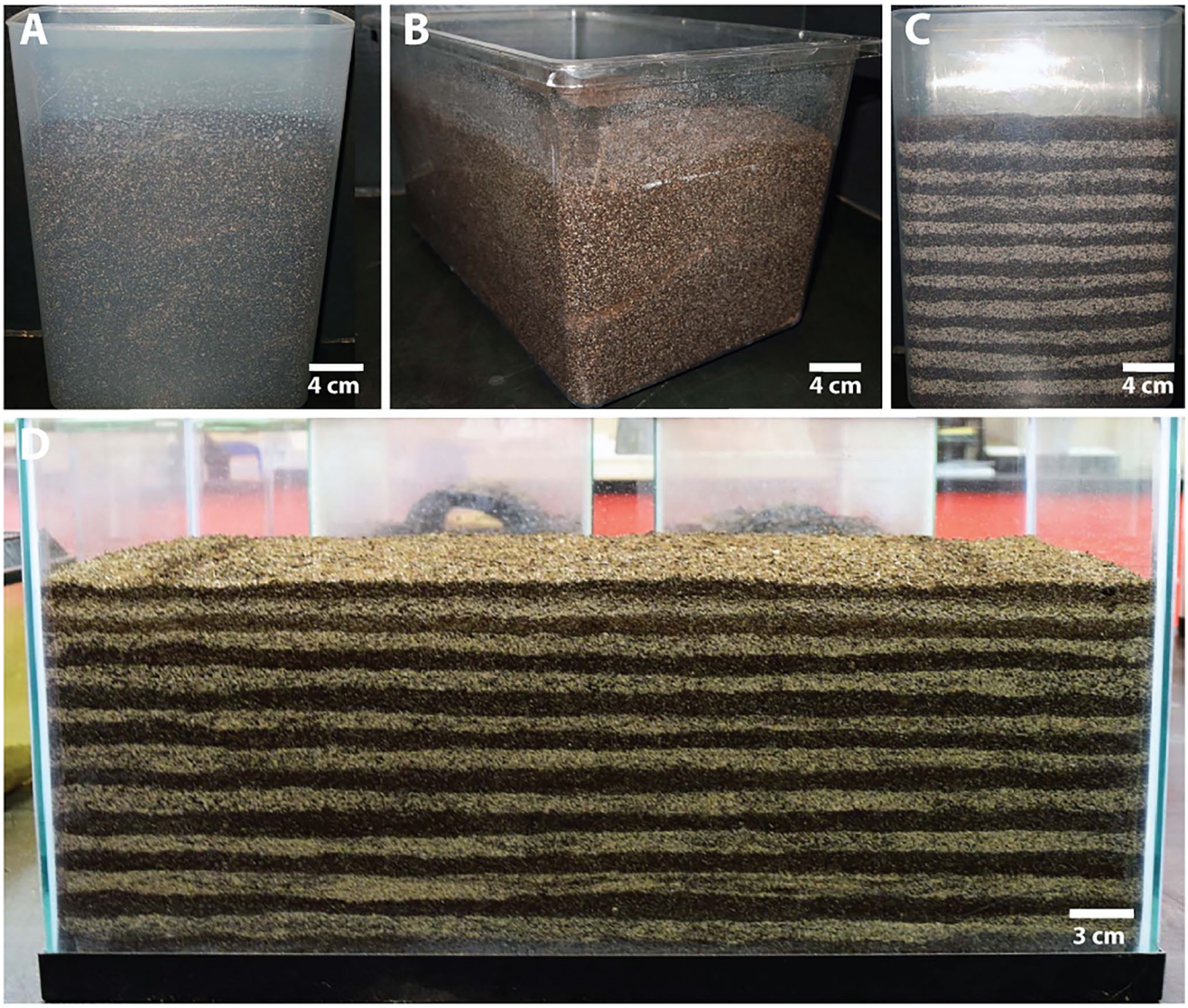

Figure 2. External view of the enclosures before the start of the experiments. (A) Experiment 1 6 L enclosure filled with massive sediment without food layers. (B) Experiment 2 14 L enclosure filled with massive sediment without food layers. (C) Experiment 3 6 L enclosure filled with layers of alternating sediment composition without food layers. (D) Experiment 4 38 L enclosure filled with layers of alternating sediment composition. The dark layers are composed of 15% sand, 50% clay, and 35% organic matter while the light layers are composed of 60% sand, 25% clay, and 15% organic matter.

with 1 cm layers of a sediment composed of 60% sand, 25% clay, and 15% organic matter (Figure 2C, Table 1). This sediment composition was selected due to its contrast with the organic-rich loam, notably its absence of organic matter and higher sand content, while still falling within the composition of normal sediment types found in natural habitats of the studied species. As with Experiments 1 and 2, two sets of eight enclosures were prepared for each species, one with and one without food layers. The contrasting layers of sediment allowed for the assessment of the amount of sediment movement and disruption produced by the larvae and adults of both species (i.e., ichnofabric) as well as the recognition of discrete biogenic structure morphologies.

In Experiment 4, a set of 12 larvae of *Z. morio* were placed in three different 38 L (50 cm × 25 cm × 30 cm) enclosures filled with 20 cm of layered sediment with the same composition and layer distribution as Experiment 3 (Figure 2D, Table 1). This experiment was designed to assess the total amount of bioturbation that *Z. morio* could produce through their life cycle as well as the morphology of the biogenic structures produced by multiple specimens interacting in the same tank. *Tenebrio molitor* was not used in this experiment due to its observed limited burrowing ability in comparison to *Z. morio*, particularly in Experiment 3.

The biogenic structures produced in each experiment were documented with photographs and

described qualitatively and quantitatively. Pupation chambers created along the sides of the enclosures were measured at the beginning of pupation and then after the adult beetle exited the chamber. Digital cameras were also used to record the specimens burrowing techniques and behaviors.

Results

General burrowing techniques

In the larval stage, both species moved continuously through the substrate using a combination of intrusion, compaction, and excavation burrowing techniques (e.g., Bromley, 1996). Entry into the substrate was predominately achieved through intrusion and compression, with little to no sediment transported to the surface. When within the sediment the larvae pushed their head forward into the substrate and then turned to either side or lifted upward to compact the substrate around the burrow (Figure 3A). Then, the appendages were used to extract and transport sediment particles back along the length of the body and deposit them along the floor of the burrow (Supporting Information Appendix A). This process resulted in open shafts and tunnels to which the larvae did not return, but did cross uncommonly. The larvae used this burrowing method until they began preparing for pupation, at which point they stopped moving and began to construct a chamber by enlarging the tunnel segment. The larvae began by scraping sediment from the walls of the developing chamber with their head and appendages (Figure 3B). The larvae then compacted the loosened sediment into the walls by moving their body in circular patterns around the perimeter of the expanding chamber (Supporting Information Appendix B). This process produced a circular to elliptical chamber in cross section, whose longest axis was similar to the length of the larva. During chamber production the larvae also closed any tunnels or shafts that led into the chamber by filling in their entry points with newly excavated sediment. Once the adult stage was reached, the new beetles exited the chamber by digging upward to the substrate surface. This was done by initially scraping the compacted sediment from the ceiling of the pupal chamber with the mouth parts and legs and then forcing their way through the overlying substrate using intrusion and excavation techniques (Figure 3C; Supporting Information Appendix C). As this was done, the substrate typically collapsed behind the adult beetle, backfilling the pupation chamber, and the exit shaft.

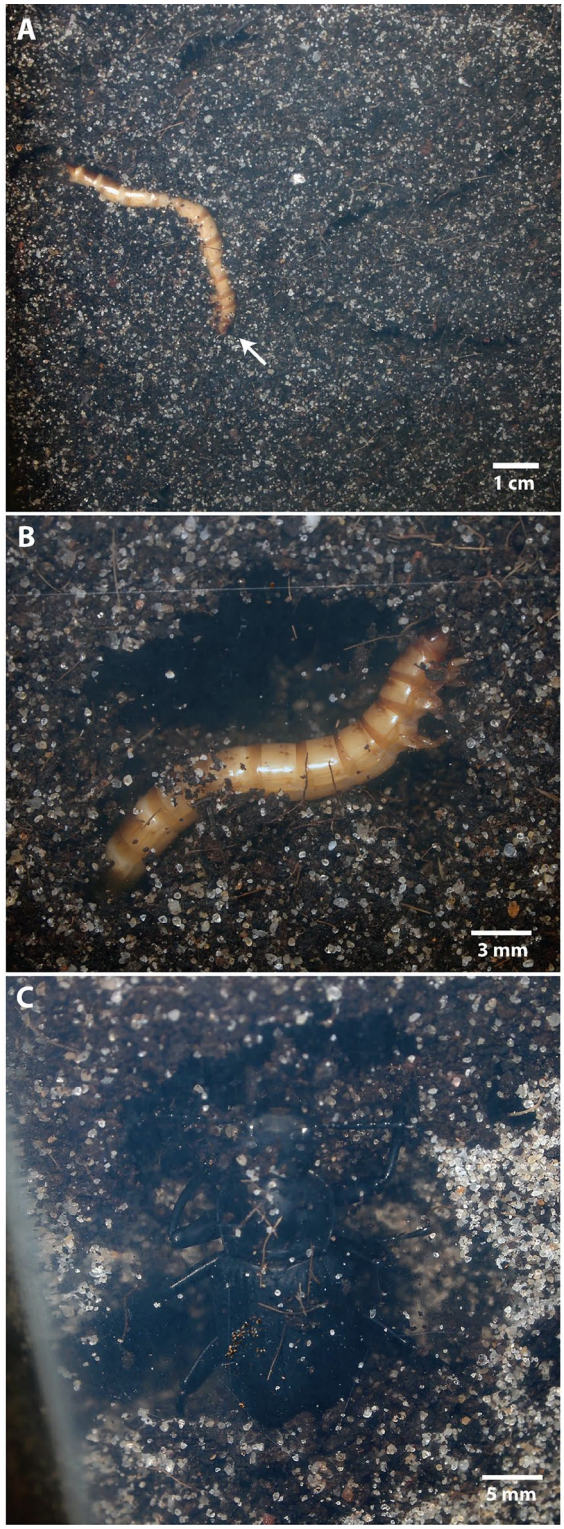

Figure 3. Burrowing techniques of darkling beetles. (A) *Zophobas morio* larva constructing an open burrow through a combination of intrusion, compaction, and excavation techniques. The head of the larva is indicated at the arrow. Continuous burrowing results in intersecting tunnels. (B) Larva of *Z. morio* using its head and appendages to produce the walls of a developing pupation chamber. (C) Adult of *Z. morio* excavating the roof of the pupation chamber with its appendages to begin movement upward to the sediment surface.

Behaviors and biogenic structures

Experiment 1 revealed high amounts of activity in the substrate by both *Zophobas morio* and *Tenebrio molitor,* primarily in the larval stage, due to the number and concentration of observed traces along the sides of the enclosures (Figure 4). While in the larval stage each species actively moved through the subsurface producing open burrows and commonly (*T. molitor*) to rarely (*Z. morio*) returned to the surface. During the larval stage, searching for food (locomotion) and deposit feeding were the main behaviors observed. Larval burrows included vertical to horizontal, open, straight to sinuous, continuous shafts and tunnels with discontinuous, thin, compacted linings recognized by their darker color from the surrounding sediment (Figure 4A,B). Some burrows had interconnected U-shaped forms at shallow depths as a result of the larvae repeatedly moving down and back up in the substrate (Figure 4B). The tunnels and shafts were not maintained, but remained open after they were abandoned until they collapsed. Larvae of *T. molitor* spent more time both on the sediment surface and in the upper portions (1–3 cm) of the substrate, thus the total density of burrows was lower and mainly restricted to the upper tier. In contrast, *Z. morio* larvae moved through the substrate more frequently, produced two to three times as many burrows in the same amount of time, and burrowed to greater depths (10–20 cm).

After 12–25 days the larvae created an open, ovoid chamber below the surface in preparation for pupation. Chamber construction took from 2 to 5 days. The pupation chamber was typically constructed at a depth of approximately 3 cm by *T. molitor* and 12 cm by *Z. morio*, but the depth varied from 1 to 20 cm (Table 2). Once the chamber was complete, the larvae curled up on the chamber floor to begin the pupation process which lasted 13–21 days (Figure 4C). When the adult stage was reached, the adult beetle exited the chamber by burrowing upward over the course of 2–5 days. The pupation chamber was always disrupted when the adult beetle exited it, but not all of the chambers were completely filled in during this process (Figure 4D). Some chambers had full collapses, some had partial collapses, and rarely a chamber would be fully preserved with either a single exit burrow or a dead larva or pupa inside. The upward movement of the adult beetle produced an indistinct, partially to fully backfilled shaft that led to the sediment surface as a result of locomotion or escape behavior (Figure 4D). Uncommonly, the adult beetle produced a new, temporary, ovoid chamber either near (~1 cm) or immediately below the sediment surface before emerging (Figure 4E). When produced, this chamber was occupied for only 1–2 days during which time the beetle remained relatively motionless.

In Experiment 2, the observed behaviors and traces produced in the 14 L enclosures were similar to those of the 6 L enclosures in all life stages (Figure 4F–H). The primary difference between the two enclosure sizes was the number of visible traces. Enclosures in Experiment 2 typically contained 10–15 identifiable traces over all four sides, whereas Experiment 1 enclosures typically contained 20–30+ (see Figure 4A,F for comparison). Much more of the burrowing activity and construction of pupation chambers occurred in the interior of the enclosure rather than along the margins and was, therefore, not visible.

The enclosures filled with layered sediment in Experiment 3 provided a better record of movement of each species, generally showing a higher amount of larval activity than the non-layered enclosures. This difference was especially evident in the upper 5 cm of the *Z. morio* enclosures (Figure 5A). *Tenebrio molitor* larvae, however, were far less active in the layered enclosures than *Z. morio* larvae (Figure 5B). *Tenebrio molitor* larvae were unable to burrow through more than one sandy layer, restricting their movement to the upper three sediment layers. *Zophobas morio* larvae, however, completely homogenized multiple layers within the layered enclosures, while also leaving evidence of continuous movement through the substrate throughout the larval stage. Pupation chambers of both species were always produced within a clay-rich layer (Figure 5C). Exit traces of the adult beetles were better defined in the layered sediment. The adult traces were characterized by the disruption of layers and series of meniscate backfills with forms varying between straight, vertical shafts that led from the pupal chamber to the surface or sub-vertical burrows that wrapped around the walls of the enclosure until the adult beetle reached the surface (Figure 5D). Once near the surface (~3 cm), as the adult beetle made its way out of the substrate, the sediment began to collapse back into void space it produced. This resulted in the production of a series of vertically nested, V-shaped laminae at the upper end of the backfilled shaft (Figure 5D).

Enclosures with layers of food in the sediment did not result in any change in the types of burrows produced, but there were changes to where the traces occurred within the enclosures (Figures 5E–G and 6). Horizontal open burrows were common along the food layers (Figures 5E and 6A). For most of the enclosures, the larva burrows most frequently occurred

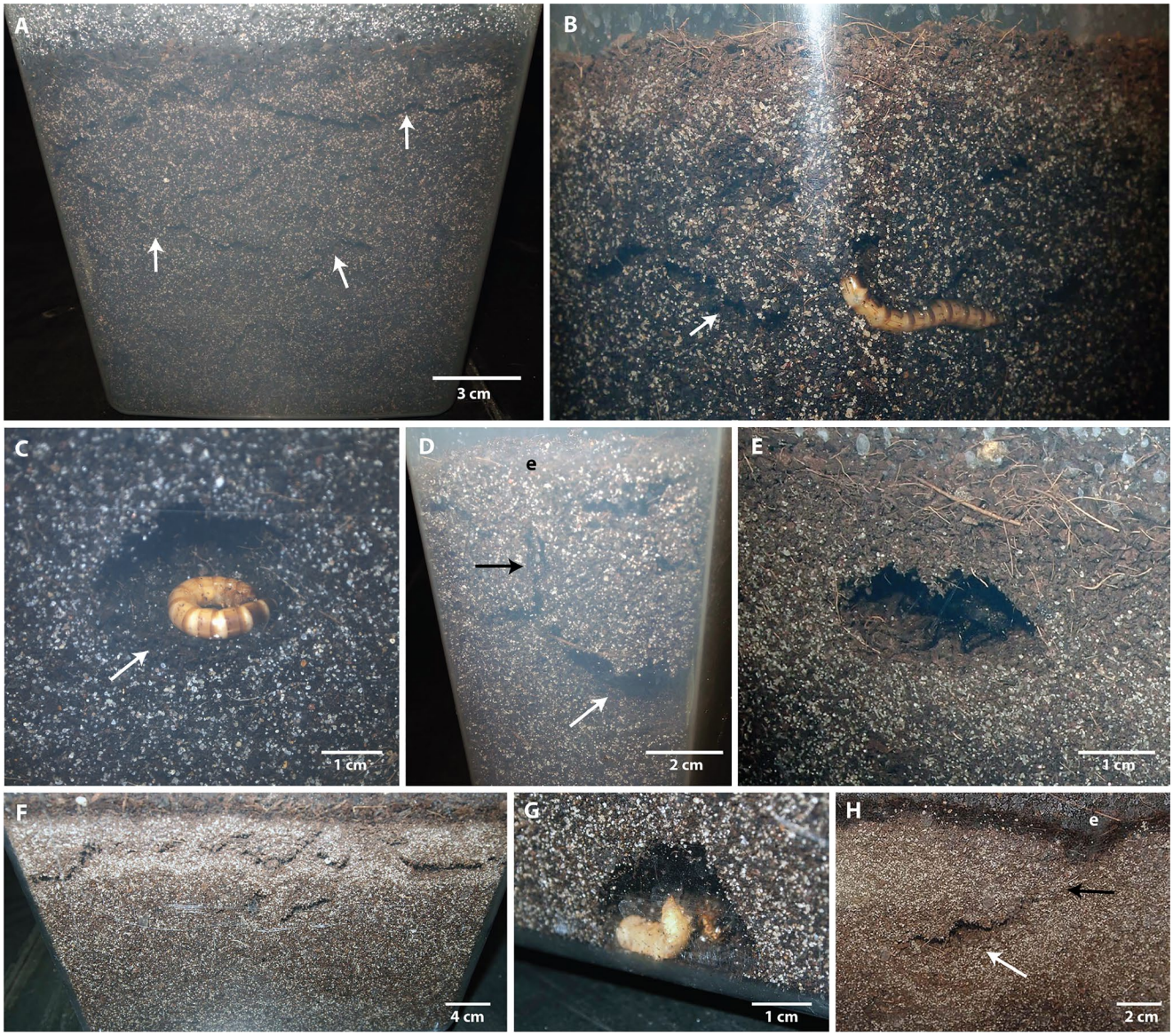

Figure 4. Traces produced in Experiments 1–2 without food layers. (A–E) Experiment 1. (A) Partially open, sinuous tunnels, and shafts (examples indicated at arrows) produced throughout the enclosure by a *Zophobas morio* larva. (B) A series of interconnected, curved to U-shaped tunnel segments produced by a *Z. morio* larva. A thin, compacted lining is visible at the arrow recognizable by the darker color of the sediment. (C) Pupation chamber of *Z. morio* with the larva in a resting state prior to pupation. (D) Partially preserved pupation chamber (at white arrow) and exit trace (at black arrow) produced by adult *Z. morio*. The surface exit is marked with an 'e'. (E) Near surface chamber produced by an adult *Tenebrio molitor* before moving to the substrate surface. (H–J) Experiment 2 with no food. (H) Assemblage of curved to U-shaped larva burrows in the upper 6 cm of the substrate. (G) Pupation chamber of *Z. morio* at the base of the enclosure. (H) Exit trace (at black arrow) produced by an adult *Z. morio* extending from a partially collapsed chamber (at white arrow) produced by the adult before exiting the substrate. Surface exit is marked with an 'e'.

near the uppermost food layer, with some burrows extending down to the lower two layers. Pupation chambers were commonly constructed at the food layers with the same morphology present in the non-food trials (Figures 5F and 6B,C). These chambers were commonly surrounded by a layer of mold or fungus extending from the food layer which formed a thin-to-thick coating along the inside surface (Figure 6D,E). Exit traces of the adult beetles possessed the same morphology as the non-food layered tanks (Figures 5G and 6F).

At the end of each trial in Experiment 4 the 38 L enclosures contained a complex assemblage of overlapping larval, pupation, and adult traces identical to those produced in Experiment 3 (Figure 7). Despite the increase in enclosure size there was still significant burrowing activity along the sides. The total visible bioturbation produced constituted an ichnofabric

Figure 5. Traces produced in Experiment 3. (A–D) Experiment 3 without food layers. (A) Sinuous shafts and tunnels and general sediment disruption features produced by a *Zophobas morio* larva in layered sediment. (B) Disruption of the upper three layers of sediment (~3 cm deep) by a *Tenebrio molitor* larva. Traces largely include sediment deformation structures including minor layer disruption and downward movement of sediment (at arrows). (C) Pupation chamber of *Z. morio*. Disrupted layers above show the path of larva movement (at arrow). (F) Exit shaft (in bracketed segment) produced by an adult *Z. morio*. The shaft is composed of backfilled sediment packets. The pupation chamber at the base of the shaft has been filled (at black arrow). The upper portion of the shaft (at white arrow) is a cone-in-cone feature where the adult beetle exited to the surface. (E–G) Experiment 3 with food layers. (E) Larva tunnel excavated along a food layer (at arrow). (F) Pupation chamber of *Z. morio* at the base of the enclosure. Disrupted layers above show the path of larva movement (at arrow). (G) Exit shaft produced by an adult *Z. morio*. The pupation chamber at the base of the shaft has been filled (at black arrow). The upper portion of the shaft (at white arrow) is a cone-in-cone feature where the adult beetle exited to the surface.

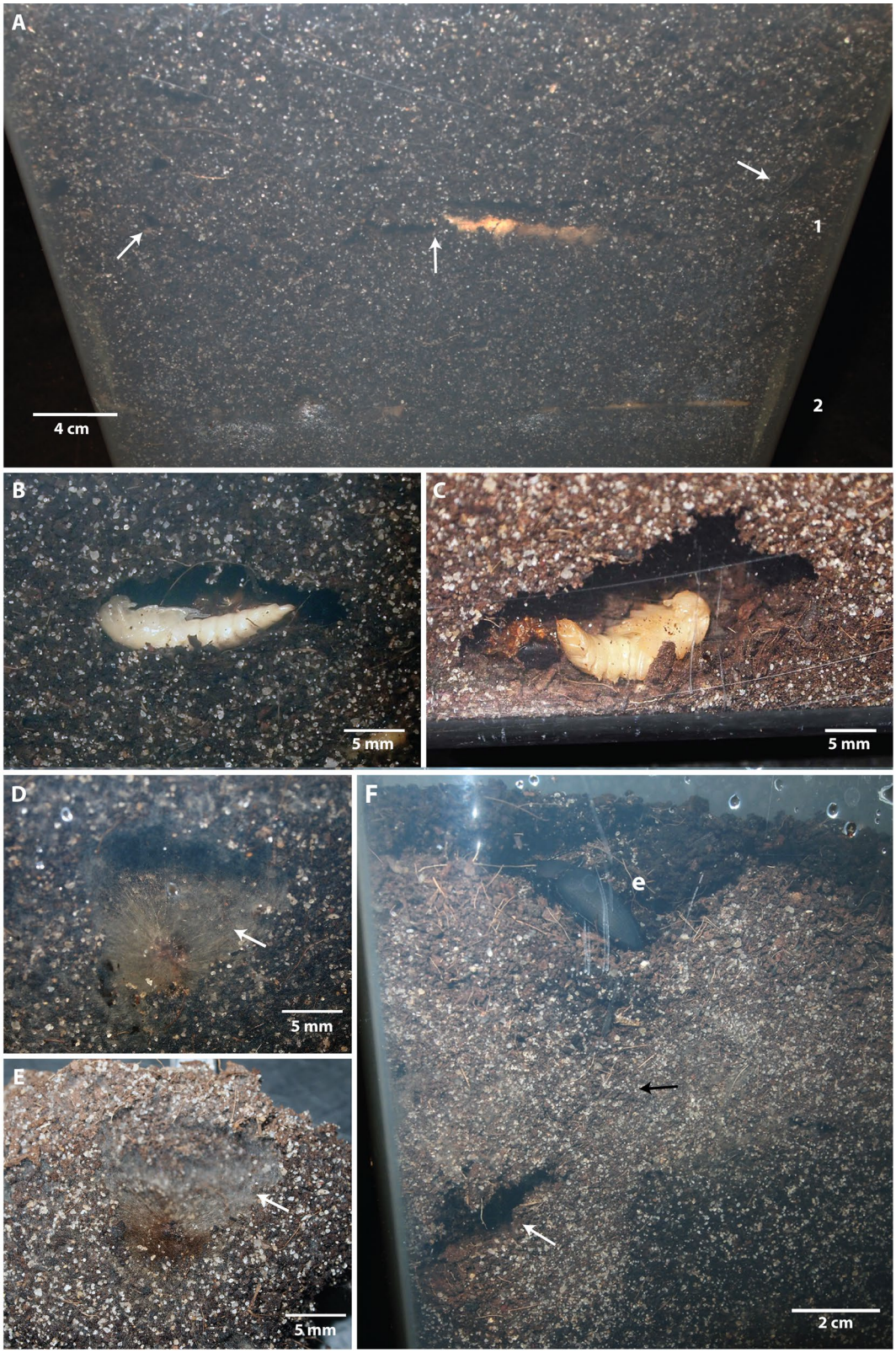

Figure 6. Traces produced in Experiments 1–2 with food layers. (A) Experiment 1 enclosure showing the occurrence of *Z. morio* larva tunnels along the upper food layer (1). (B) Pupation chamber of *Z. morio* along food layer in Experiment 1. (C) Pupation chamber of *Z. morio* at the base of the enclosure in Experiment 2. (D) Open chamber along food layer in Experiment 1 with the interior surface coated in fungus (at arrow). (E) Excavated partial pupation chamber (at arrow) showing fungal coating along the interior surface. (F) Partially preserved pupation chamber (at white arrow) and exit trace (at black arrow) produced by the adult *Z. morio* visible on the surface. Surface exit is marked with an 'e'.

index of 2–3. The majority of this bioturbation was produced in the larval stage within 20–30 days. Most of the larval burrows collapsed by the end of the experiment when all adults had emerged, but were still preserved as recognizable sediment disruption features. Pupation chambers were largely filled in

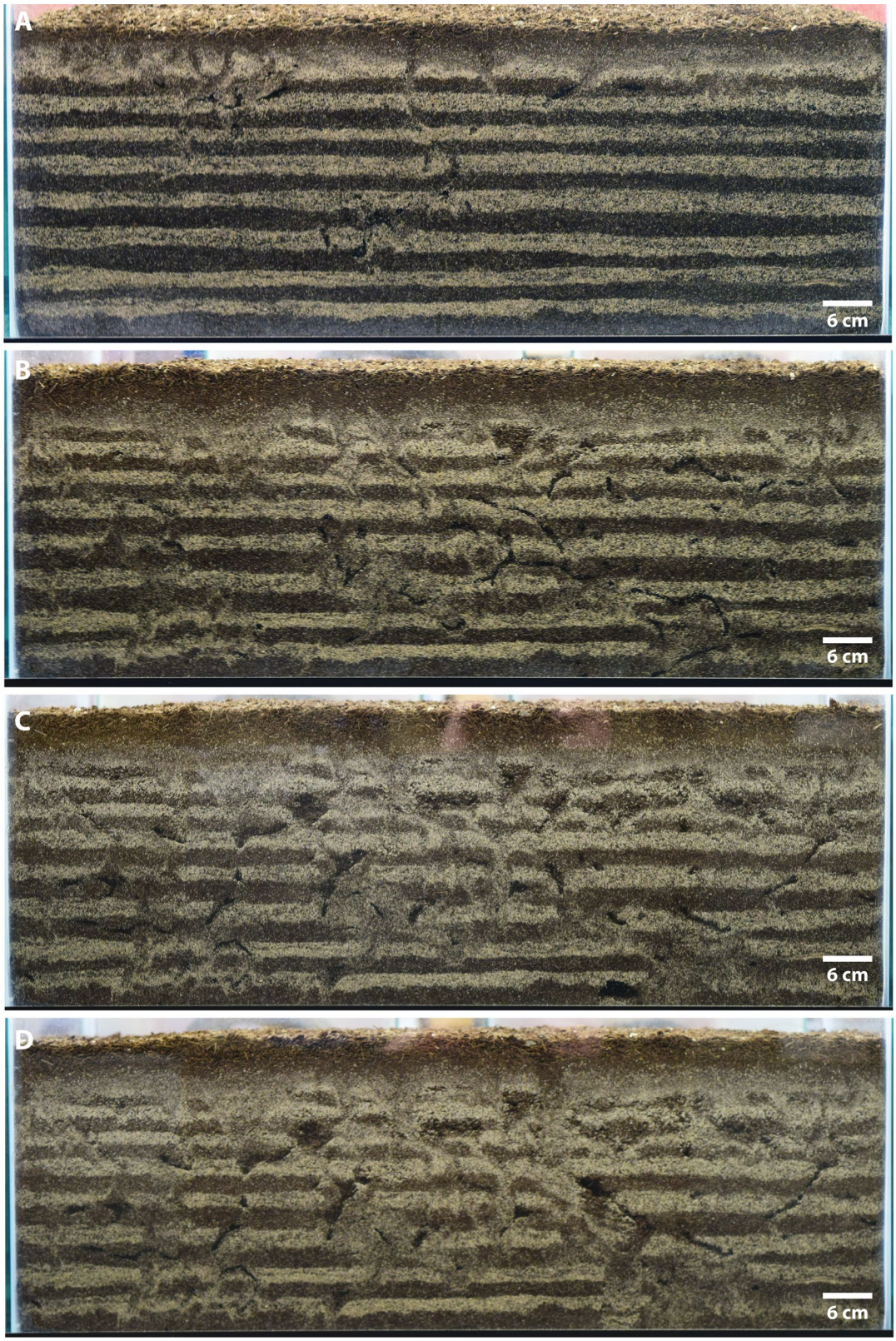

Figure 7. Overall disruption of sediment over time by 12 specimens of *Zophobas morio* in a 38 L tank trials of Experiment 4. (A) Day 1. (B) Day 20. (C) Day 60. (D) Day 72 (end of trial).

when the adult emerged, but the fill was distinct from the surrounding sedimentary fabric. Adult exit burrows included straight to sinuous backfilled shafts.

General morphology of darkling beetle traces

Larvae burrows

The traces produced by the larvae of each species were similar in morphology. Both species produced straight to sinuous, horizontal to vertical or sub-vertical, continuous, unlined to a very thin compacted lining, open to passively filled tunnels and shafts with circular to elliptical cross sections (Figure 8A–C). Burrows of *T. molitor* had a mean width of 3 mm and burrows of *Z. morio* a mean width of 5 mm (Table 2). False branching was present, resulting from the larvae crossing a previously created burrow. Burrows consisting of interconnected, U-shaped segments were also produced which were shallow, elongate, and sometimes repeating. The abundance and density of larval traces decreased with depth in both species, being highest at depths <5 cm for *T. molitor* and <10 cm for *Z. morio*.

Pupation chambers

Both species produced pupation chambers with similar morphologies, but different sizes (Table 2). In both species, the pupation chambers were ovoid with smooth interior sides, often surrounded by a thin zone of visibly modified sediment, and the long axis parallel to the substrate surface (Figure 8D–F). The width-to-height ratio of the chambers had some variation, but generally they had a lower height than width (Table 2). The mean width-to-height ratio for all *T. molitor* pupation chambers was 1.83 ($n = 5$) and 1.93 ($n = 24$) for *Z. morio* chambers. The depth of the chambers varied from shallow (1 cm) to deep (20 cm), but were generally produced in the lower half of the enclosures (10–20 cm). Pupation chambers were partially or completely filled when the adult exited the chamber. Complete chambers were most commonly preserved when the larva or pupa died before metamorphosis.

Adult traces

Adult traces primarily consisted of burrows produced when leaving the pupation chambers; these had little variation in morphology between each species except for size. Exit traces were vertical to sub-horizontal, open to actively filled shafts and tunnels (Figure 8G,H). Mean exit tunnel width was 1 cm for *T. molitor* and 1.5 cm for *Z. morio* (Table 2). In enclosures filled with massive sediment, the actively filled exit burrows rarely had a recognizable difference from the surrounding sediment. In the layered enclosures, the exit burrows were filled by arcuate to meniscate backfill packets, approximately 5 mm thick, and the offsetting and truncation of sediment layers (Figure 8G,H). The exit shaft typically widened at the top producing a funnel shape where the adult exited the substrate and moved to the surface (Figure 8G). Adult beetles rarely produced an irregular, unlined, ovoid chamber at the top of the exit shaft just below the sediment surface (Figure 4E). Adult beetles did not burrow into the substrate after coming to the surface.

Discussion

Trace morphology, body morphology, and behavior

The lack of variation between the trace morphologies of each species was largely due to their similar body morphologies and behaviors (e.g., Bromley, 1996; Genise, 2017). The two key differences between the burrows of each species were the larger size, greater abundance, and the greater total depth of burrows of *Zophobas morio*, especially in the larval stage. These differences can be linked to the larger body size of *Z. morio* at all life stages as well as their more robust appendages in the larval and adult stages which made them more effective burrowers.

Each life stage of both species produced different morphologies of biogenic structures, which could be distinguished as those of the larval, pupation, and adult life stages when in the same enclosure at the end of each trial. The trace morphologies were different in each life stage due to the different behaviors exhibited during each life stage as well as the significant changes that occurred in body morphology (e.g., Bromley, 1996; Genise, 2017). During the larval stage, searching for food (locomotion) and deposit feeding were the main behaviors observed. The continuous, open shafts, and tunnels produced by the larvae were present in a large portion of the available substrate when discrete food layers were not provided. In trials with food layers, larvae burrows were more concentrated along these layers. This observation explains why the tunnels, while left open, were not revisited and were distributed throughout the enclosures. Based on direct observations during the experiments, the larvae appeared to be in a continuous search for food throughout the enclosure until construction of a pupation chamber as also observed in other, similar

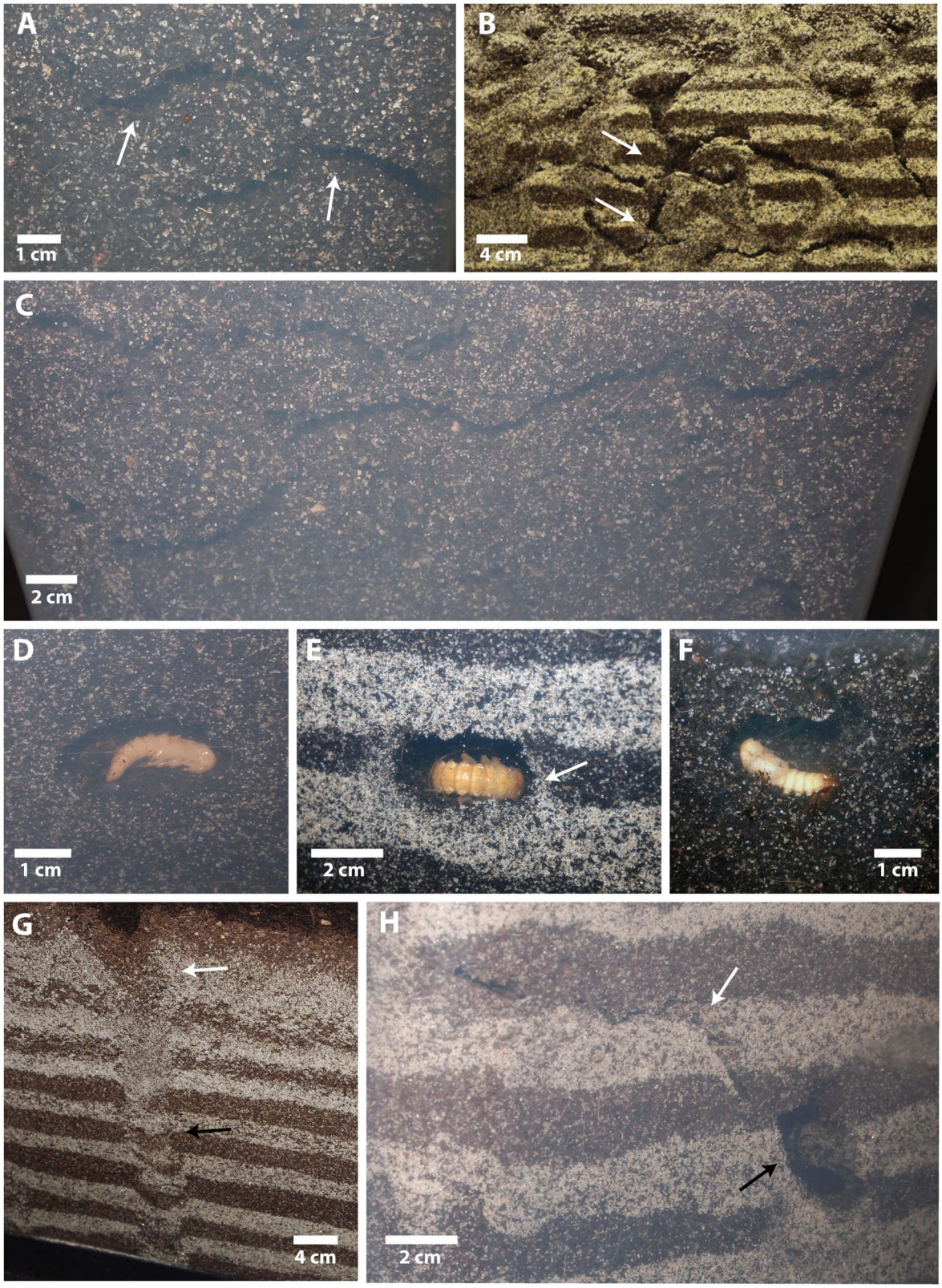

Figure 8. Standard trace morphologies produced in Experiments 1–4. (A) Typical curved, horizontal traces produced by *Zophobas morio* larvae (at arrows). (B) False branching pattern occurring from tunnels overlapping (*Z. morio*) (at arrows). (C) Sinuous tunnels produced by *Z. morio* larvae. (D) Pupation chamber produced by *Z. morio*. (E) Pupation chamber from Experiment 3, produced between sand layers by *Z. morio*. (F) Typical pupal chamber produced by *Tenebrio molitor*. (G) Backfilled adult exit shaft produced by *Z. morio* (at black arrow) terminating near the surface with a series of nested, cone-in-cone structures (at white arrow). The pupation chamber at the base has been filled. (H) Exit tunnel (at white arrow) produced by adult *Z. morio* which remained partially open with the pupation chamber still intact (at black arrow).

experiments with beetle larvae (e.g., Counts & Hasiotis, 2009; Smith & Hasiotis, 2008).

Pupation chambers were constructed at the end of the larval stage by both species and marked the end of the food searching behavior by the larvae. Once the chambers were complete, the larvae entered the pupal stage and remained in the chamber until the adult stage. No other associated behaviors were observed during this stage, as the pupae remained relatively motionless in the chamber. The length of the long axis of the pupation chamber was similar to the length of the larva that excavated it as well as the pupa that would occupy the chamber. The length of the chambers was always greater than the height, leaving enough room for the pupa to lie within it lengthwise. The pupae were always positioned on their 'back' and oriented horizontally in the chamber, matching the orientation of the long axis of the chamber.

The traces produced by adult specimens were distinct from those of the larvae due to the significant changes in body morphology which resulted in different burrowing techniques as well as a change in the primary behavior. The adult beetles produced one main type of trace, which was the result of moving upward to the surface of the substrate. This activity was comparable to an escape behavior (e.g., Bromley, 1996; Buatois & Mángano, 2011). The large, non-radial body morphology and longer, more robust appendages of the adult beetles resulted in wider tunnels than the tunnels produced in the larval stage. These burrows were also less likely to remain intact and open than the larva burrows since they were mainly vertical in orientation and extended to the less stable surface layers (e.g., Bromley, 1996).

Trace morphology and environmental conditions

The inclusion of food layers in the substrate did concentrate the occurrence of beetle larva tunnels along the layers, reinforcing the link between larva burrow production and feeding. This response was expected given that the main behavior of the beetle larval stage is searching for food (Marshall, 2018). Larvae of other beetles with feeding habits similar to those of *T. molitor* and *Z. morio* should, therefore, exhibit similar behavioral response to food concentrations in sediment and soil.

The availability of space in the experiments did not change the morphology of the traces produced. This outcome is similar to other laboratory-based experiments conducted with burrowing terrestrial animals (e.g., Bowen & Hembree, 2014; Catena & Hembree, 2014; Dzenowski & Hembree, 2014; Hembree, 2009, 2013; Hembree et al., 2012; Hils & Hembree, 2015). The main difference between the smaller and larger enclosures with a single specimen was the reduced concentration of burrows throughout the substrate when available space was increased. The larvae tended to move through the substrate the same amount, regardless of the space available. As a result, the overall disruption of sediment was lower in the larger tanks. When the number of specimens was increased to 12 in the 38 L tank, however, this resulted in a high concentration of burrows in the substrate. Given these results, the basic morphology of the traces produced through the beetle's life cycle could match those produced under natural conditions despite their spatial constraints.

The substrate composition was also found to be an important control on the morphology and presence of traces produced by the beetles (e.g., Bromley, 1996). *Tenebrio molitor*, for example, was much less likely to travel through the loose, sandy substrate in the layered trials, preferring the more cohesive, finer-grained substrate instead. In sandy substrates, *T. molitor* generally did not produce traces at all. *Zophobas morio*, in contrast, did travel through the sandier substrates, suggesting that they are more likely to be found in a larger range of substrate consistencies. A notable consideration, however, is that the *Z. morio* larvae always made their pupation chambers within the finer-grained layers (Figure 5C,F), likely because of increased structural stability of that sediment composition.

Preservation potential

The traces produced by the beetles in each life stage were found to have different levels of durability at the end of the experiments. The durability of the traces is an indication of their potential for preservation in the fossil record, with the caveat that there are many processes between trace production and lithification of the host sediment. The shafts and tunnels produced by the beetle larvae had varying levels of durability. The smaller burrows of the *Tenebrio molitor* larvae, for example, were more prone to collapse than the larger burrows of the *Zophobas morio* larvae. Upon collapse, the tunnels were difficult to recognize in the massive sediment, but still obvious in the layered sediment due to the disruption of the layers and sediment mixing (Figures 5A,B and 7). Those larval burrows that did remain open,

particularly the larger burrows of *Z. morio*, have the potential to be filled in by precipitating minerals from groundwater (e.g., Bromley, 1996; Savrda, 2007). Infilling by sediment from above is unlikely since the burrows did not have a maintained connection to the sediment surface.

Pupation chambers produced by each beetle species were structurally similar and each was equally likely to destroy and infill the chamber upon exiting. Due to this tendency for the chambers to collapse as a result of the adult beetle exiting, pupation chambers for each species are much less likely to be preserved by passive infilling of sediment, precipitation of minerals, or other sedimentary processes that would preserve the entire chamber. These pupation chambers are more likely to be preserved as an actively produced disruption of the host sedimentary fabric, especially if the process of exiting the chamber causes a different sediment type to infill the chamber (e.g., Bromley, 1996; Genise, 2017). Complete and intact chambers were observed at the end of several experiments, but occurred most often when the specimens died in the chamber in the larval or pupal stage. In addition, rare examples of mold and fungus growing around and within the chamber during trials with food layers caused the chambers to be less prone to collapse, thus, increasing their preservation potential. When chambers remain intact and open, there is an opportunity for preservation during the lithification process (Bromley, 1996). Open chambers allow for the precipitation of minerals in the void space, preserving the full morphology of the chamber (Savrda, 2007). The uncommon occurrence of intact pupation chambers remaining in the substrate at the end of these experiments is interesting because ichnofossils interpreted as the pupation chambers of beetles are quite abundant in Mesozoic and Cenozoic paleosols (Genise, 2017). It is possible that the destruction of pupation chambers upon emergence observed in these experiments may be associated with darkling beetles, whereas other beetles have more resilient pupation chambers.

Traces produced by adult beetle have the best chance of preservation if there is variation in sediment composition between the chamber and the surface to enhance the appearance of backfilling. Adult beetle traces were most evident in the layered tank experiments where the exited chambers infilled and the vertical shaft consisted of nested, arcuate to meniscate backfill packets. The exit tunnels that remained open after the adult specimen reached the surface would also have the opportunity to be preserved if passively infilled since they were connected to the surface (e.g., Bromley, 1996).

Comparison of observed beetle traces to continental ichnotaxa

The traces produced by *Tenebrio molitor* and *Zophobas morio* during their different life stages in this study have a number of features that can help in the recognition of beetle traces in the fossil record. Larvae traces of both species would be preserved as horizontal to sub-vertical, sinuous, passively filled burrows within paleosols, likely concentrated in areas where organic matter was present as a food source (e.g., Hembree & Hasiotis, 2007). There would likely be overprinting of larval traces from multiple individuals passing the same areas over an extended period of time. Larval traces may also be recognized by disruption and homogenization of the host sediment fabric, but many tracemakers can contribute to the general bioturbation of paleosols. The larvae traces produced by the beetles in these experiments are similar to open burrows such as *Planolites* and *Palaeophycus* (Hall, 1847; Nicholson, 1873) (Figures 4A,B, 5A,E, and 8A–C). *Planolites* has many similarities to the larval traces produced herein, consisting of cylindrical to elliptical, unlined, straight to curved, horizontal to subvertical burrows. However, *Planolites* are defined as actively filled burrows (Pemberton & Frey, 1982), whereas the traces produced by the two larval beetle species in these experiments were always left open. *Palaeophycus* from continental deposits have previously been attributed to beetles (Krapovickas et al., 2010) and the passively filled, cylindrical burrows produced in these experiments seemingly match the descriptions of *Palaeophycus*, but while compacted linings were observed along the length of some burrows, most lacked a clear lining. *Palaeophycus* is also described as having intermittent branching (Hantzschel, 1975). The branching tunnels produced in this experiment were directly observed as false branching (the branches were produced by cross-cutting and were not revisited), but after their production they may appear as simultaneous branching since they were left open and connected. Branches in the beetle larvae tunnels were vertically oriented and did not widen at the branch points since the specimens did not return to pass back through previously created tunnels. Overall, the beetle larvae traces shared characteristics with both of these common ichnogenera that can occur in continental environments, but do not share all of the characteristics with either of them. These

differences suggest that there is no described ichnogenus that fully represents the morphology of the burrows produced by the larval specimens in these experiments.

Complete pupation chambers produced in these experiments would appear as ovoid chambers with smooth interior sides, with the long axis of the chamber oriented parallel to the substrate surface, and surrounded by a zone of disrupted sediment. If preserved in this way, especially if it lacked an obvious emergence hole or burrow, it is likely that the beetle died in the chamber. Partially preserved pupation chambers, the most common type observed in these experiments, would have an exit shaft leading from the chamber. The morphology of the chambers themselves would vary due to the disruption caused when the adult specimen exited the chamber. Partially collapsed pupation chambers usually maintained their morphology in the portions that were not destroyed, so they may appear as hemispherical chambers if preserved.

The complete pupation chambers produced in this study can be compared to *Fictovichnus gobiensis* (Johnston et al., 1996) (Figures 5F, 6B,C, and 8D), *Rebuffoichnus casamiquelai* (Roselli, 1987) (Figures 5C and 8E,F), and *Pallichnus dakotensis* (Retallack, 1984) (Figure 4C,G). *Fictovichnus gobiensis* is described as an ellipsoidal to ovoid chamber with a horizontal to subhorizontal orientation, rounded terminations at either end of the long axis, and a smooth outer surface (Genise, 2017; Johnston et al., 1996). *Pallichnus dakotensis*, in contrast, is a nearly spherical chamber with a thin outer lining of dark clay and organic matter and one end of the chamber is marked by an irregularly circular disruption (Genise, 2017; Retallack, 1984). *Rebuffoichnus casamiquelai* is a subcylindrical to oblate chamber with a horizontal to subhorizontal orientation and a smooth inner surface that may be perforated by a round, terminal, or medial hole. Occurrences of these ichnospecies have been previously interpreted as pupation chambers of beetles (Catena et al., 2017; Genise, 2017; Guerrero-Arenas et al., 2018; Johnston et al., 1996; Zapata et al., 2016). There are several other ichnogenera attributed to pupation chambers (see Genise, 2017), however, these possess complex surface features or thick linings not observed in the chambers produced in these experiments and are not comparable. Different pupation chambers produced by the darkling beetles in the experiments could be attributed to any one of these three ichnospecies given the irregularity of the overall form of the chambers, varying from circular, to ovoid, to subcylindrical. This is especially true of those chambers that partially collapsed when the adult burrowed upward to the substrate surface.

The adult beetle traces produced in these experiments would be expected to be preserved as vertical, open burrows leading to the surface, with the overlying sediment infilling the lower sections of the burrow or as vertical, backfilled burrows. Subvertical, passively filled burrows are also expected to be preserved with a similar morphology to those produced in the larval stage but with a larger diameter and lower sinuosity.

Adult traces produced in these experiments were comparable to *Taenidium*, *Planolites, Skolithos,* and *Conichnus* (Heer, 1877; Myannil, 1966). Regular and elongate exit shafts produced in the adult stage were compared to *Taenidium,* being vertical, straight, unbranched, unlined, and with a meniscate backfill (Figures 5D and 8G). Exit traces with this morphology were best observed in enclosures with layered substrates. The less common, sub-vertical traces produced in massive substrates that led from the chamber to the surface were similar to *Planolites* as described for the larval traces, but were often partially passively filled near the surface (Figure 8H). More rarely, partially open, vertical shafts extended to the surface similar in form to *Skolithos* (Figure 4D). Finally, *Conichnus* is most similar to the exit structures in these experiments that were produced near the sediment surface, often at the upper termination of backfilled burrows or passively collapsed burrows (Figures 5D,G and 8G). *Conichnus* is described as a vertical cone with a rounded basal termination that is passively filled, sometimes with spreite, occurring in both terrestrial and marine environments (Frey & Howard, 1981; Myannil, 1966). These near surface, vertical exit traces were characterized by overlain V-shaped layers of passively infilled sediment from above as the specimen moved to the surface. Often the opening to the surface would form a cone-shaped depression as well.

Conclusions

Neoichnological studies such as this one are important for understanding the composition of ancient terrestrial ecosystems. Because terrestrial environments, particularly soils, often have poor preservation potential for body fossils of soil invertebrates, being able to better interpret the ichnological record for soil-dwelling organisms is critical. The high diversity and abundance of beetles in soil ecosystems since the late Mesozoic makes a good understanding of their

trace fossil record vital for reconstructing past terrestrial ecosystems.

Zophobas morio and *Tenebrio molitor* were good candidates for understanding the typical traces produced by soil-dwelling beetles because they are generalist species with distinct body morphologies in each life stage. Other types of soil-dwelling beetles with similar morphologies and behaviors through each life stage would be expected to create traces comparable to those produced in these experiments. Importantly trace morphology did not vary significantly between the two species, but was instead controlled by the body morphology and behaviors of each life stage. The distinct trace morphologies produced in each life stage would allow them to be distinguished as larval, pupal, or adult traces in the fossil record. This is important because each life stage is linked to specific behaviors and it prevents overestimation of biodiversity based on the presence of multiple trace morphologies. In this case a single species may produce several different ichnogenera through its life cycle in a relatively limited area.

Available space was found to have little impact on trace morphology, indicating that the observed traces were not a product of spatial constraints and similar morphologies should be expected under natural conditions. Concentration of organic material (i.e., food layers) resulting in the more frequent occurrence of larvae burrows along specific horizons whereas concentration of coarser-grained sediment (i.e., sand) excluded most burrowing activity. Such observations could be useful in interpreting some of the causes of the spatial distribution of different traces.

Neoichnological experiments in both the laboratory and the field are necessary to improve our understanding of the different types of traces that animals can produce under a variety of conditions. These observations form the basis for our interpretation of the potential tracemakers and behavioral and environmental significance of ichnofossils. The traces produced by the two species of darkling beetles during their different life stages in these experiments were comparable to several different ichnogenera including *Palaeophycus, Planolites, Fictovichnus, Rebuffoichnus, Pallichnus, Taenidium, Skolithos,* and *Conichnus*, yet there were many significant differences. When viewing these and similar ichnogenera in terrestrial environments, these experiments support the interpretation of beetles as a potential trace maker if preserved in a paleosol and depending on their morphological details. Careful study would still be required to rule out other potential trace makers. However, neoichnological studies such as this help to improve the understanding of the array of trace morphologies that different types of soil animals can produce. Importantly, direct observation of trace production from start to finish is incredibly useful in informing interpretations of the connections between trace morphology, behaviors, and trace makers. Due to the poor body fossil record of beetles, improving our ability to recognize beetles as potential trace makers in continental environments allows for better interpretations of terrestrial ecosystems through time, and could also provide critical information on the evolution of beetles and their behaviors through time.

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